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ORIGINAL INVESTIGATION

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Urinary excretion of *N*-acetyl-*S*-allyl-L-cysteine upon garlic consumption by human volunteers

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Abstract *N*-Acetyl-*S*-allyl-L-cysteine (allylmercapturic acid, ALMA) was previously detected in urine from humans consuming garlic. Exposure of rats to allyl halides is also known to lead to excretion of ALMA in urine. ALMA is a potential biomarker for exposure assessment of workers exposed to allyl halides. It is not known whether garlic consumption can lead to urinary concentrations of ALMA which may interfere with biological monitoring of exposure to allyl halides by determination of urinary ALMA. Therefore, this study was undertaken to determine the cumulative excretion and the excretion kinetics of ALMA in urine of humans consuming garlic. Six human volunteers were given orally two garlic tablets, each containing 100 mg garlic extract (each representing 300 mg fresh garlic). Three of the volunteers consumed additional garlic after the garlic tablet intake. Urine samples were collected up to 24 h after the intake of the garlic tablets. ALMA was identified in the urine using gas chromatography-mass spectrometry (GC-MS) and determined quantitatively with a limit of detection of 0.10 μg/ml with gas chromatography with sulphur selective detection. The total amount of ALMA found in urine of volunteers who consumed two garlic tablets was 0.43 ± 0.14 mg ($n = 3$). In the urine of the three volunteers who consumed not only two garlic tablets but also additional fresh garlic, a significantly higher amount of ALMA was excreted in the urine, 1.4 ± 0.2 mg ($n = 3$). The elimination half-life of ALMA, estimated from urinary excretion rate versus time curves, was 6.0 ± 1.3 h ($n = 5$). One volunteer, who ate additional garlic, showed an irregular elimination profile and was excluded from this estimation. The highest urinary concentration of ALMA found in this study was 2.2 μg/ml. In a preliminary biological monitoring study of exposure in workers with potential exposure to allyl chloride (AC) up to the occupational exposure limit of 1 ppm (8-h TWA), we recently found urinary ALMA concentrations up to 4 μg/ml. Based on the results presented here, we conclude that garlic consumption is a potential confounder when monitoring human exposure to allylhalides and other chemicals leading to ALMA excretion when ALMA is used as a biomarker of exposure.

Key words Allyl chloride · Biological monitoring · Garlic · Mercapturic acid · Urine

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Introduction

N-Acetyl-L-cysteine-*S*-conjugates or mercapturic acids (MAs) are frequently used as biomarkers of electrophilic chemicals in biomonitoring and toxicological studies (Vermeulen 1989; Van Welie et al. 1992). Electrophilic compounds may react either spontaneously or enzymatically with the endogenous tripeptide glutathione (γ-Glu-Cys-Gly; GSH) forming glutathione conjugates. So-called MAs are formed from these GSH-conjugates by removal of the γ-glutamyl- and glycine moieties by γ-glutamyl transpeptidase and dipeptidases, respectively followed by *N*-acetylation of the resulting L-cysteine conjugates by cysteine conjugate *N*-acetyl transferase. MAs have been analysed in rat and human urine samples with chromatographic methods such as high pressure liquid chromatography (HPLC), gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) (Van Welie et al. 1992). Allylmercapturic acid (ALMA) has

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been measured as urinary metabolite in rats treated with allyl halides (Kaye et al. 1972; De Rooij et al., to be published) and has been identified in urine of humans consuming garlic (Jandke and Spiteller 1987). Allyl halides such as allyl chloride have been found to be mutagenic in the *Salmonella typhimurium* mutagenicity assay (Eder et al. 1982).

In garlic bulbs (*Allium sativum*) numerous sulphur-containing compounds have been identified (Block 1992), including (+)-*S*-2-propenyl-L-cysteine-*S*-oxide (alliin), the precursor of allylthio-allylsulphoxide (allicin), and two other L-cysteine sulphoxides, namely, *S*-(E)-1-propenyl- and *S*-methyl-L-cysteine sulphoxide. Besides these compounds at least three sulphur-containing γ-glutamyl peptides have been identified in garlic (Mütsch-Eckner et al. 1992a), namely γ-L-glutamyl-*S*-trans-1-propenyl-L-cysteine, γ-L-glutamyl-*S*-allyl-L-cysteine and γ-L-glutamyl-*S*-allylthio-L-cysteine. These products are thought to be the sulphur and nitrogen stores in the garlic bulb. After consumption of garlic, several sulphur-containing compounds, such as diallyl disulphide, diallyl sulphide and dimethyldisulphide (Bartzatt et al. 1992) and also MAs, such as *N*-acetyl-*S*-2-carboxypropyl-L-cysteine (CPMA) and *N*-acetyl-*S*-allyl-L-cysteine (ALMA) (Jandke and Spiteller 1987), are excreted in urine. CPMA was also identified in urine of humans consuming onions (Jandke and Spiteller 1987).

In a preliminary biomonitoring study, designed to assess human exposure to allyl chloride, we found urinary ALMA excretion in humans who did not have contact with allyl chloride before urine collection. However, they did report consuming garlic on a regular basis. Up till now no data have been reported on amounts of ALMA excreted in human urine after consumption of garlic. The aim of this study was therefore to elucidate whether garlic consumption can lead to urinary concentrations of ALMA which might interfere with biological monitoring of humans exposed to allyl halides and other chemicals leading to ALMA excretion.

Materials and methods

Chemicals and synthesis of reference compounds

Benzylmercapturic acid (BEMA) and *N*-acetyl-L-cysteine were obtained from Janssen Chimica (Beerse, Belgium) and creatinine was from Baker (Deventer, The Netherlands). All chemicals used in this study were of the highest purity available. Garlic tablets (Kwai) were from Lichtwer Pharma (Berlin, Germany) and contained 100 mg garlic extract, extracted from 300 mg garlic bulb. Allylmercapturic acid (ALMA) was synthesized from allyl bromide and *N*-acetyl-L-cysteine as described elsewhere (Kaye et al. 1972). Chemical shifts in NMR (Van Bladeren et al. 1980) and the mass spectrum (Jandke and Spiteller 1987) of synthetic ALMA were in accordance with literature data. Ethereal diazomethane was generated from *N*-methyl-*N*-nitroso-urea and 50% w/v potassium hydroxide in diethyl ether according to Vogel (1967).

Extraction of ALMA from urine

To an aliquot of 3 ml urine, 1.5 µg BEMA was added as internal standard. The urine was acidified to pH 1.5 (±0.5) by adding 0.1–0.3 ml of 2 M hydrochloric acid and subsequently extracted twice with 3-ml portions of ethyl acetate. A clear phase separation was obtained after centrifugation during 0.5 h at 2500 *g*. The ethyl acetate layers were combined and evaporated to dryness at 50°C under a stream of nitrogen. The residue was dissolved in 0.5 ml methanol and subsequently methylated with ethereal diazomethane. After this, the organic solvents were evaporated again and the residue was redissolved in 0.2 ml ethyl acetate. Aliquots of 1 µl of the ethyl acetate layer were used for GC and GC-MS analysis.

Gas chromatographic sulphur selective analysis of ALMA

Analysis of the methylated extracts were carried out on a Hewlett Packard (HP) 5890 GC equipped with a flame photometric detector (FPD) used in the sulphur mode. Calibration of the detector was performed with a double logarithmically plotted calibration curve, since an FPD has a non-linear response (Sevcik 1976). Chromatography was carried out using a Chrompack Sil 19-CB WCOT capillary column with a length of 25 m, an internal diameter of 0.25 mm and a stationary phase thickness of 0.2 µm. The column was operated at a head pressure of 150 kPa, resulting in a carrier flow of 2.5 ml/min measured at 50°C. Helium was used as carrier gas. The temperature of the injector and the detector were held at 250°C. Injection of 1-µl aliquots took place in splitless mode, with a purge time of 0.75 min. When high concentrations of ALMA (> 10 µg/ml) were measured injections were made in split mode (split ratio 1/15). The oven temperature was programmed from 50°C (1 min) to 170°C on a ramp of 20°C/min. The temperature of 170°C was held constant for 3 min and there after a second ramp of 10°C/min was performed until 250°C. This temperature was held constant for 5 min.

Gas chromatographic mass selective analysis of ALMA

The identity of ALMA in urine of volunteers consuming garlic was confirmed by GC-MS analysis of the methylated urine extracts using a HP 5890 GC equipped with a HP 5970 mass selective detector (MSD). Chromatography was performed using a Chrompack Sil 5-CB WCOT capillary column with dimensions as described above. The temperature of the injector and the transfer line were held at 250°C. The oven was programmed from 50°C (1 min) to 140°C with a rate of 20°C/min. This temperature was left constant for 3 min and thereafter the oven was further heated to 250°C at a rate of 8°C/min. The final temperature was left constant for 5 min. Ions were generated by electron impact ionization at an electron energy of 70 eV. When the GC-MSD was used in scan mode, ions were scanned with a mass to charge ratio of 75–500. Ions were detected at a potential of 2.4 kV and an emission current of 20 mA. When the GC-MSD was used in selective ion monitoring (SIM) mode, the ions at m/z 144, 158 and 176 were detected at low mass resolution at a potential of 2.4 kV and an emission current of 20 mA.

Human volunteer experiments

A group of six young adults were asked to volunteer in the garlic consumption experiment. Two garlic tablets were taken on 1700 hours. Urine fractions were collected completely in separate fractions, just before the intake of the tablets and for up to 24 h thereafter. Urine samples were stored at 4°C before delivery at the laboratory, where the volume of the urine fractions was measured

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and where 50 ml samples were stored at −18°C until analysis. Urinary creatinine concentrations were determined using the Jaffe reaction (Tietz 1986). All volunteers were asked to complete a questionnaire on age, gender, body weight, other possible exposures and lifestyle factors such as smoking.

Calculations

Urinary excretion parameters were calculated from semilogarithmic urinary excretion rate versus time curves as described in literature (Van Welie et al. 1991). Semilogarithmic urinary excretion rate (dQ/dt) versus time curves were constructed for the urinary ALMA excretion in every volunteer. Whenever necessary the elimination-rate versus time profiles were corrected for creatinine excretion. The urinary elimination rate constant (k_{el}) of ALMA was calculated by linear regression of the terminal linear parts of these curves by the formula: slope $= -k_{el}/2.303$. Linear regression was performed using Statworks from Macintosh. The corresponding urinary half-life of elimination ($t_{1/2}$ of elimination) was calculated from the elimination rate constant (k_{el}) according to the equation: $t_{1/2}$ of elimination $= 0.693/k_{el}$. The standard deviation given by Statworks for the correlation of the slope was used to calculate the standard deviation of the half-life. Total cumulative ALMA excretion was calculated from the ALMA excretions in the collected urine fractions. Data were statistically analysed by using the unpaired Student's *t*-test. The level of statistical significance was set at $p < 0.05$. Data are presented as average ± sd.

Results

Evaluation of the analytical procedures

Extraction of ALMA from spiked urine (30 µg/ml) was found to yield 98 ± 9% ($n = 4$). Calibration curves were linear in a range from 0.1 to 10 µg/ml (splitless injection) and from 10 to 100 µg/ml (split injection). The detection limit using GC with sulphur selective detection was found to be 0.10 µg/ml urine (S/N = 3/1). With GC-MSD a detection limit of 0.010 µg/ml urine (S/N = 3/1) was found when the MSD was used in SIM mode at m/z 158. The response of the ion at m/z 158 was linear in a range from 0.040 µg/ml (S/N = 10/1) to 4.0 µg/ml urine. The stability of ALMA was tested in spiked urine samples. No significant decrease in ALMA concentrations was found within 3 months when urine was stored at −18°C. Methylated urine extracts were tested for stability for 12 months and found to be stable in this period when stored at room temperature.

Excretion of ALMA by volunteers consuming garlic

Six volunteers, two males and four females, participated in the garlic consumption experiment. The age of the volunteers was in a range from 20 to 27 years, their body weights ranged from 60 to 90 kg. The excretion of ALMA upon intake of two garlic containing tablets could be confirmed by full mass spectra in urine samples with high concentrations of ALMA (not shown). At lower concentrations of ALMA, the excretion of ALMA was confirmed by the mass fragmentograms of the fragments at m/z 144, 158 and 176 at the retention time of synthetic ALMA (9.42 min), see Fig. 1.

The concentrations ALMA found in the urine fractions in this study ranged from the detection limit of 0.1 µg/ml up to 2.2 µg/ml. Three volunteers reported the consumption of additional garlic during the study. Their average cumulative excretion of ALMA was higher (1.4 ± 0.2 mg) than that of the other volunteers (0.43 ± 0.14 mg). From the concentrations of ALMA found, elimination rate versus time (dQ/dt) profiles were constructed for each volunteer. From these data, the averaged elimination half-life of ALMA excretion was determined to be 6.0 ± 1.3 h ($n = 5$). One volunteer, who consumed additional garlic, was excluded from this determination due to an irregular elimination profile. In Fig. 2A and B, two characteristic elimination

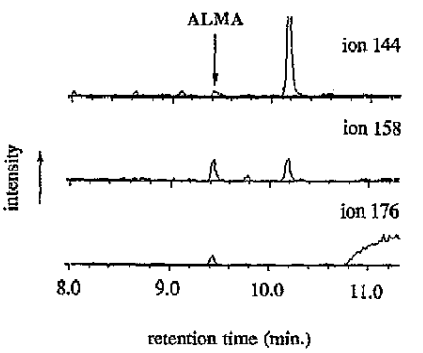

Fig. 1 Mass fragmentogram of a methylated urine sample of a human volunteer after consumption of two garlic tablets containing 100 mg garlic extract. The excretion of allylmercapturic acid (*ALMA*) is confirmed by mass fragmentograms of the ions of the methylester at m/z 144, 158 and 176. ALMA is indicated by the *arrow* at retention time 9.4 min

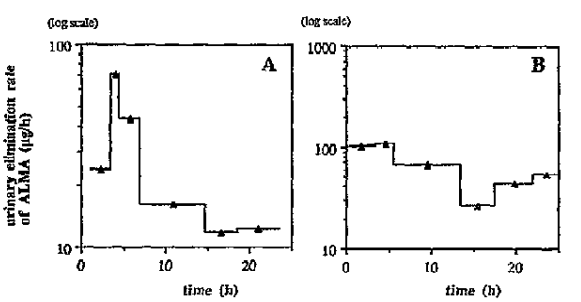

Fig. 2A, B The elimination rate (µg/h) versus time (h) pattern of ALMA in two volunteers after consumption of two garlic tablets at time zero. **A** The excretion rate versus time pattern of volunteer 1. **B** The excretion rate versus time pattern of volunteer 4, who consumed additional garlic after intake of two garlic tablets

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profiles are shown for a volunteer consuming only the garlic tablets and a volunteer who consumed additional garlic during the study, respectively.

Discussion

The aim of this study was to elucidate whether garlic consumption could lead to concentrations of ALMA in the urine which might interfere with biological monitoring procedures in humans exposed to allyl halides and other chemicals leading to ALMA excretion. It is known that exposure of rats to allyl halides leads to the excretion of ALMA in urine (Kaye et al. 1972).

The data presented here clearly indicate that it is possible to analyse, qualitatively and quantitatively, ALMA in human urine samples by GC with mass selective (MSD) as well as with sulphur selective detection (FPD). Sulphur selective detection (FPD) was about ten times less sensitive than GC-MS in SIM-mode.

In all six volunteers who participated in this study, we could clearly demonstrate the excretion of ALMA in urine after consumption of natural garlic or of garlic tablets. The excretion of ALMA in urine was confirmed with GC-MSD with three or more characteristic ions (Jandke and Spiteller 1987) (Fig. 1). Identification of ALMA in extracts of very large volumes of human urine after garlic consumption was described previously (Jandke and Spiteller 1987). However, the levels at which ALMA was excreted were not established. We measured urinary ALMA concentrations ranging from the detection limit of the assay (0.1 µg/ml urine, GC-FPD) up to 2.2 µg/ml urine.

In one volunteer, consuming additional garlic, we found an irregular elimination profile from which determination of the urinary half-life of ALMA could not be performed (Table 1). The additional garlic uptake could have disturbed the elimination profile. In the other volunteers the mean urinary elimination half-life of ALMA was 6.0 ± 1.3 h ($n = 5$). The urinary elimination half-life of ALMA found in the present study is slower than the elimination half-life for ALMA excretion recently found in animals orally treated with *S*-allyl-L-cysteine (e.g. for rats $t_{1/2}$ 1.63–2.33 h, Nagae et al. 1994).

Among the many sulphur-containing compounds found in garlic (Block 1992), γ-glutamyl-*S*-allyl-L-cysteine (GAC) is the most likely precursor of ALMA. γ-Glutamine is first hydrolysed from GAC by γ-glutamine-transpeptidase, resulting in the formation of *S*-allyl-L-cysteine. This L-cysteine conjugate is subsequently *N*-acetylated to the mercapturic acid by cysteine conjugate *N*-acetyl transferase (Chasseaud 1976; Commandeur et al. 1995). The content of GAC in garlic is dependent of the site of garlic production (Mütsch-Eckner et al. 1992b). As a consequence, it is difficult to

Table 1 Excretion data on ALMA by the volunteers participating in the garlic consumption experiments

Volunteer	Totally excreted (mg)	Estimated half-life (h) (average ± sd)
1	0.47	4.9 ± 1.0
2	0.55	7.5 ± 4.4
3	0.28	7.3 ± 1.3
4	1.40[a]	5.2 ± 0.62
5	1.53[a]	ND[b]
6	1.17[a]	5.0 ± 1.8

[a] Consumed additional garlic during the study
[b] Irregular elimination-time profile

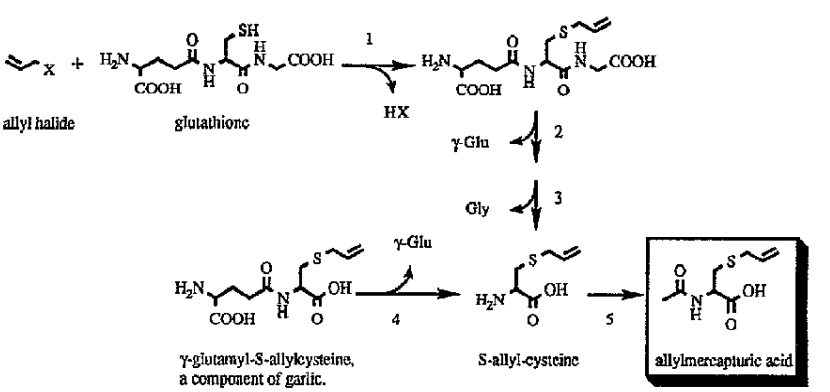

Fig. 3 Proposed mechanism by which ALMA is formed from γ-glutamyl-*S*-allylcysteine or from glutathione (*GSH*) conjugation with an allyl halide. Step 1 may be catalysed by GSH-*S*-transferases or formed nonenzymatically. The GSH-conjugate is subsequently hydrolysed to *S*-allyl-cysteine by γ-glutamyl-transpeptidase and cysteinylglycinase (step 2 and 3). γ-Glutamyl-*S*-allylcysteine may also be hydrolysed (step 4) with γ-glutamyltranspeptidase to form the corresponding allyl cysteine. In the final step (5), allyl cysteine is *N*-acetylated by cysteine-conjugate-*N*-acetyltransferase to form ALMA. *X* halide, *γ-Glu* γ-glutamyl, *Gly* glycine

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calculate exactly how much of GAC is excreted as ALMA in the urine. However, assuming that 1% of the dry weight of garlic bulbs represents GAC (Mütsch-Eckner et al. 1992b), we estimate that approximately 10% of the GAC is excreted as ALMA over the first 24 h following oral administration. In this calculation we assume that the complete dry weight products were incorporated in the garlic tablets. Only data obtained in volunteers who consumed only garlic tablets were used for this calculation. This rather high percentage may be explained by the high bioavailability of *S*-allyl-L-cysteine, the precursor for ALMA, in rats (98.2%, Nagae et al. 1994).

Mercapturic acids are often used to assess human exposure to electrophilic compounds (Vermeulen 1989; Van Welie et al. 1992). In the case of allyl halides, nucleophilic substitution of the halide atoms by glutathione and subsequent processing to a mercapturic acid may also lead to the excretion of ALMA into urine. In a preliminary biological monitoring study of exposure in workers with potential exposure to AC up to the occupational exposure limit of 1 ppm (8-h TWA), we recently found urinary ALMA concentrations up to 4 µg/ml (data not shown). In the present study, we found urinary ALMA concentrations between 0.1 and 2.2 µg/ml. Based on the results presented here, we conclude that garlic consumption may interfere significantly and therefore is a potential confounder when monitoring human exposure to allyl halides and other chemicals leading to ALMA excretion with ALMA as a biomarker of exposure.

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